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Biological Effects of TMPRSS2/ERG Fusion Isoforms in Human Prostate Cancer

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It has been established that 15-80% of prostate cancers harbor the TMPRSS2/ERG fusion gene depending on the clinical stage, with 40-60% of surgically treated cancers containing the gene fusion. Thus it is the single most common molecular alteration in prostate cancer and as such is a critical target for diagnostic testing and novel therapies. However, there is currently very little information about the biological functions of TMPRSS2/ERG fusions and the signaling pathways affected by this fusion in prostate cancer. Understanding the underlying mechanism by which this gene fusion promotes prostate cancer initiation and progression will assist us to better predict prognosis of patients and prospectively develop novel therapeutic methods for prostate cancer. We propose to extend these studies to primary prostate epithelial cells and fully transformed prostate cancer cells to further our understanding of the biological affects of the fusion isoforms in prostate cancer cells.

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**Introduction**

Recent reports from several independent research groups have shown that fusion of the androgen-regulated TMPRSS2 gene and the ERG gene exists in the majority of surgical treated prostate cancer patient tissue samples. In our previous study, we identified 8 alternatively spliced fusion isoforms in clinically localized prostate cancer. Type III and Type VI are the two most frequent isoforms, and expression of the Type VI isoform is associated with aggressive clinical behavior in clinically localized prostate cancer. Results of experiments presented in this application using immortalized PNT1A prostate cells indicate that Type III and Type VI, isoforms, alone or in combination, promote cell proliferation, migration, and invasion. Using quantitative RT-PCR arrays we have identified candidate mediators of these phenotypic effects. We propose to extend these studies to primary prostate epithelial cells and fully transformed prostate cancer cells to further our understanding of the biological affects of the fusion isoforms in prostate cancer cells.

Our hypothesis is that the TMPRSS2/ERG fusion gene isoforms have variable biological activities in prostate epithelial cells (PrEC) that can contribute to tumor initiation, such as increased proliferation, decreased apoptosis and/or senescence and that these fusion isoforms can also promote cancer progression in prostate cancer cells (VCaP), via increased growth rate, invasion and motility. The extent of these activities depends on the isoforms of the fusion gene expressed. Furthermore, specific downstream targets of the TMPRSS2/ERG fusion isoforms, including, but not limited to Syk and MTSS1 tumor suppressor genes we have identified in our Preliminary Studies, mediate these phenotypic effects.

**Body**

Accomplishments

Task 1. Evaluation of biological affects of TMPRSS2ERG fusion Type III, VI, III+VI expression in human primary prostate epithelial cells PrEC and VCaP cancer cells *in vitro* (Months 1-12)

a. We will make retroviruses expressing TMPRSS2/ERG fusion Type III, VI and GFP only (control) using pBMN-IRES-EGFP vector system. (Months 1-3);

In the process of cloning, we discovered 5 alternative splicing exons of ERG gene from prostate cancer patients and/or VCaP cells, among which exon 72bp and exon 81bp are frequently identified, while the other three, exon 61bp, exon 218bp and exon 152bp have not been reported before. Since Type III + 72 and VI + 72 are the relative abundant variants in prostate cancer patients (see publication), we made retroviruses expressing TMPRSS2/ERG fusion Type III+72; VI+72 and GFP using pBMN-IRES-EGFP vector system.

b. Infect PrEC cells with retroviruses expressing Type III, VI, III+VI and GFP control under optimal conditions; infect VCaP cells with Type VI and GFP carrying viruses; verify the expression of fusion isoforms by GFP imaging, Western blot and real-time PCR before further experiments. (Months 3-6)

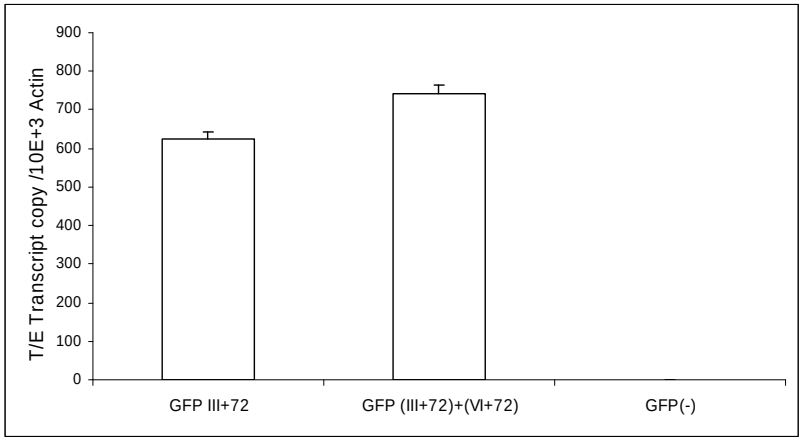
All cell groups were made and the expressions of fusion genes were confirmed.

c. PrEC cells *in vitro* studies: We will examine the number of passages before loss of viability, proliferation rate (by cell counting), apoptosis (using TUNEL) and senescence (by senescence associated β–galactosidase assay) among all groups to investigate the effects of fusion proteins on cell growth and immortalization.(Months 6-12)

Using retroviral constructs from Task 1a we stably expressed TMPRSS2/ERG fusion proteins in PrEC. Three groups of PrEC cells were generated: vector negative control; Type III+72 and Type (III+72) + (VI+72) groups. The fusion gene transcript levels are shown in Figure 1A. PrEC cells expressing the Type III+72 or Type (III +72) + (VI+72) fusion had significantly increased proliferation rate ($P<0.01$, t-test, day 9, Fig1B)

when compared to vector controls. We are still in the process of confirming TUNEL and senescence associated β–galactosidase assay results among all PrEC groups at this time point.

A

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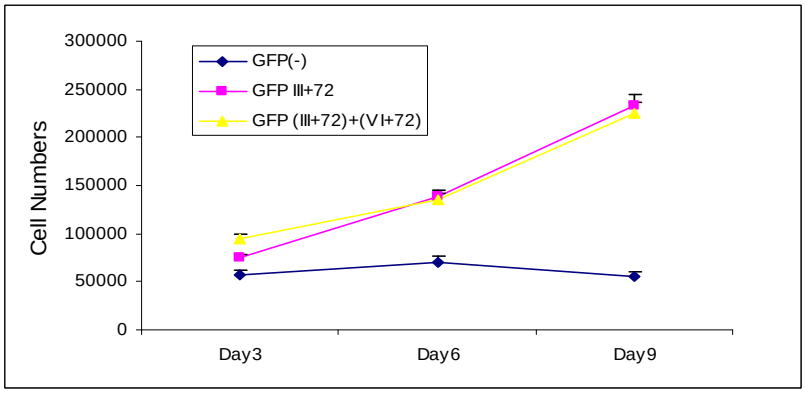


Figure1. **TMPRSS2/ERG fusion isoforms increase PrEC cell growth**

1A Expression level of fusion in PrEC cells were evaluated by real-time PCR, normalized to β-actin.

1B. The growth of the three groups of PrEC cells expressing TMPRSS2/ERG fusion type III+72, (III+72) + (VI+72) or empty vector were measured by using a Coulter counter. Cells (2.5 X 104) of each cell group were plated in 35mm dishes in complete medium. Cells were trypsinized and counted at day 3, 6 and 9 in triplicate. Mean +/- standard deviation is shown.

d. VCaP cells *in vitro* studies: Cell proliferation, motility, invasion and colony formation in soft agar will be performed to VCaP GFP cells and VCaP+Type VI cells.(Months 6-12)

VCaP cells were stably infected with Type VI and GFP carrying viruses. There's no significant growth rate difference found between these two groups. These experiments were repeated three twice. We did not carry out the comparative studies in VCaP cells of motility, invasion and colony formation in soft agar because of technique issues: (1) VCaP cells never cover the wounded area when doing the scratch assays even after 6 days of culture; (2) VCaP cells cannot pass 8uM pore in Matrigel chamber after 72h in culture; (3) VCaP cells didn't form colonies in soft agar after 2 weeks.

e. We will design shRNA against ERG and potentially specifically against fusion Type III, and validate the silencing function of these shRNA at both RNA and protein level; we will generate lentivirus carrying these validated shRNA and infect VCaP cells. VCaP cells with knockdown of ERG expression will be analyzed by experiments similar to those described above.

We designed single-stranded primer oligos targeting sequences around the fusion III junction site by using Invitrogen website BLOCK-iT™ RNAi Designer (http://rnaidesigner.invitrogen.com/rnaiexpress/). We also used a reported si scramble target sequence generate a negative control shRNA. VCaP is a common used prostate cancer cell line with endogenous expresses of these fusions. The phenotypic changes when knocking down the endogenous fusion will assist us to better understand the function of these fusion isoforms in cancer cells. As shown in Figure 2A, these lentiviruses carrying shRNA can efficiently knock down more than 60% Type III fusion expression tested in 293t cells by western blot using anti-V5 antibody. Transcriptional level of

fusion was also assessed from VCaP cells by quantitive real-time PCR using the primers recognizing all type of fusion. Similarly, ~60% knock down efficiency was found in VCaP SiIII group compared to control VCaP Si (-) group using scramble shRNA and plain VCaP cells, Figure 2A. Proliferation assay was performed. Decreased cell growth was found in VCaP SiIII group compared to control group ($P<0.01$, day8), indicating these fusion isoforms can affect cell proliferation. Experiments were repeated three times with essential same results.

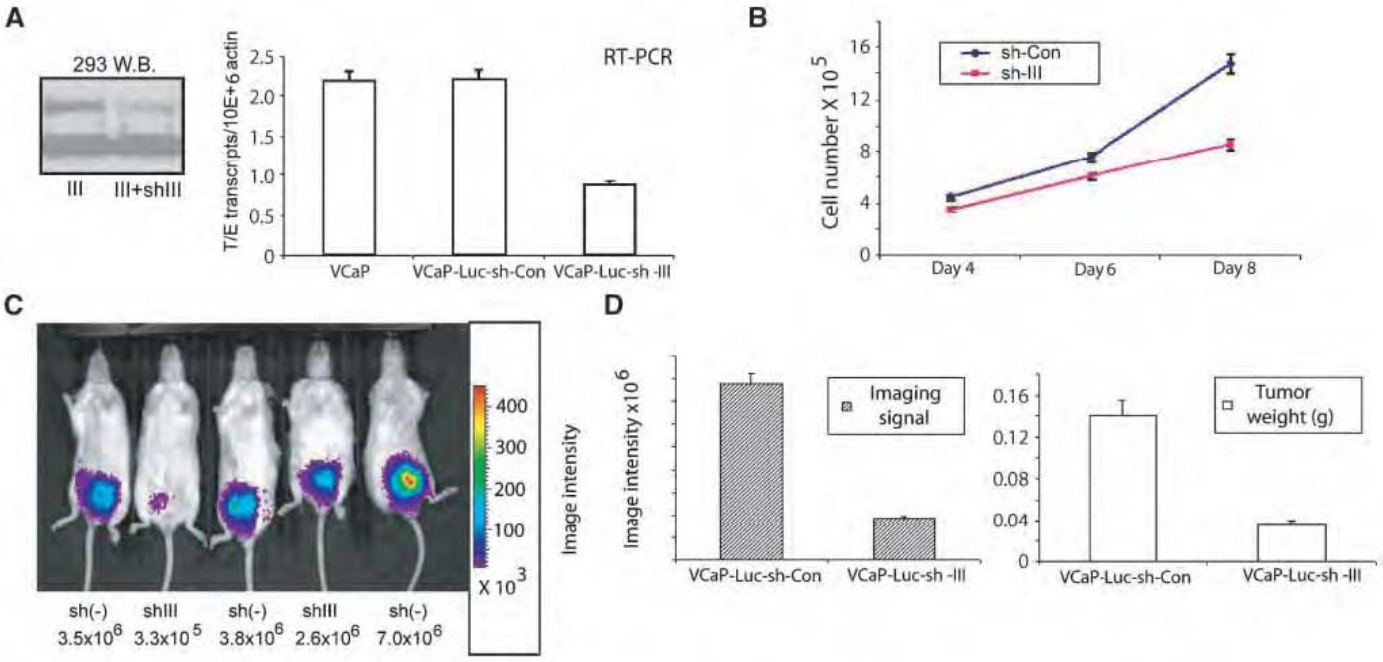


Figure 2 Knockdown Type III fusion in VCaP cells decreased cell proliferation in vitro and tumor weight in vivo.

A. Knockdown Type III fusion efficiency was more than 60% tested by western blot using anti-V5 in 293t cells transiently overexpressed with Type III fusion and infected by Lentivirus carrying shRNA against Type III fusion. β-actin in lower panel was for loading control. Expression level of fusion in VCaP cells, VCaP cells si negative control or VCaP cells si III were evaluated by real-time PCR, normalized to β-actin. B. Cell growth curve was done at day 4, 6, 8 by comparing VCaP si (-) and Vcap SiIII cell numbers, both cell groups were infected with retrovirus carrying luciferase. C. Luciferase imaging of tumor growth in live animal by IVIS imaging system at time point of 4 weeks. The left side 2 mice were orthotopically injected by VCaP Si III cells; the right side three mice were VCaP Si(-) cells. D. Luciferase imaging signal was collected at time point of 4 weeks after orthotopic

Task 2. Evaluation of biological function of TMPRSS2/ERG fusion in VCaP cells *in vivo* (Months 6-18)

a. We will infect VCaP cell expressing luciferase (VCaP-Luc) with retrovirus carrying GFP-Type VI or GFP only, cell will be selected and maintained by both G418 and puromycin, and pooled cells will be used for mice injection. If cells are viable, we will also infect VCaP-Luc cells with lentivirus carrying shRNA against ERG as well as negative control virus. (Months 6-12)

b. We will inject VCaP-Luc and VCaP-Luc+Type VI cells as well as potential VCaP and VCaP-shRNA cells orthotopically into the prostates of nude mice; 20 mice in each group will be used; we will use IVIS imaging system to monitor the tumor growth and metastasis and sacrifice the mice at an optimal time point. Primary tumor size and weight will be recorded and a full necropsy will be performed to look for metastasis.(Months 10-

12)

c. Statistical analysis will be performed on tumor growth and metastasis among different groups. Mice tumors will be submitted for histology; proliferation marker (Ki-67) and CD31 immunohistochemistry (for quantiating microvessel density), as well as apoptosis (TUNEL assay) will be carried out on tumor slides and quantitated. (Months 13-18)

Since we didn't find any growth, motility and invasion difference in vitro when overexpressing Type VI in VCaP cells, we didn't perform orthotropic injection to mice as planned in Task 2a and 2b. While in vitro data showed that knock down of the fusion expression can decrease the VCaP cell growth; we intended to test the tumor growth in vivo in orthotropic mice model. Collaborated with Dr. David Spencer at Baylor College of Medicine, we have successfully generated two stable cell lines, VCaP-Luc-SiIII and VCaP-Luc-Si (-). By using IVIS imaging system, we can monitor the tumor growth in live animals (Figure 2C). Luciferase imaging can be seen at 2 weeks from most of mice. At time point of 4 weeks, 2 out of 20 (10%) mice in Si (-) group and 6 out of 20 (30%) mice in si III group didn't form tumor. The average imaging signal was 3912837 in Si (-) group compared to 937417 in Si III group which is only about 1/4 of si (-) group. We terminated the experiment at this time point, and collected all tumors. As shown in Figure 1D, tumor weight in Si (-) group was more than 4 fold compared to these in Si III group, consistently with the imaging data. Therefore, markedly reduced cell growth proved both in vitro and in vivo strongly suggests TMPRSS2/ERG fusion isoforms have an important role in regulating cell growth. We were able to collected total of 14 mice tumor at the end point. Histology studies will be performed within the first 6 month of second calendar year. Because of very small tumor collected in SiIII group, we may need to repeat the same orthotropic injection again if larger tumor tissue is needed for further studies.

* (Task 3 and 4 will be completed in the 2nd year; Task 5 will be finished in 3rd year)

**Key Research Accomplishments**

1. We discovered 5 alternative splicing exons of ERG gene from prostate cancer patients and/or VCaP cells, among which exon 72bp and exon 81bp are frequently identified, while the other three, exon 61bp, exon 218bp and exon 152bp have not been reported before. Since Type III + 72 and VI + 72 are the relative abundant variants in prostate cancer patients, we cloned these two isoforms. Our studies indicated that TMPRSS2/ERG fusion isoforms can promote tumor initiation and progression and that these activities depend on the expression of various isoforms of the fusion gene and ERG splicing exons.
2. TMPRSS2/ERG fusion gene isoforms can promote cell growth in prostate epithelial cells (PrEC).
3. The induction of specific shRNA against Type III fusion in VCaP cells dramatically decreased cell proliferation in vitro and tumor weight in vivo, which provide us a basis of potential therapeutic approach.

**Reportable Outcomes**

Pleiotropic Biological Activities of Alternatively Spliced TMPRSS2/ERG Fusion Gene Transcripts
Jianghua Wang, Yi Cai, Wendong Yu, Chengxi Ren, Shantu Dixit and Michael Ittmann

Cancer Res 2008; 68: (20). October 15, 2008

Pleiotropic Biological Activities of Alternatively Spliced TMPRSS2/ERG Fusion Gene Transcripts
Jianghua Wang, Yi Cai, Wendong Yu, Chengxi Ren, Shantu Dixit and Michael Ittmann

Abstract, Baylor 6th Cancer Symposium, Nov 20th, 2008

**Conclusion**

In the first year, we have been systematically studied the TMPRSS2/ERG fusion isoforms in Pca. In summary, we reported 5 alternative splicing exons of ERG gene, three of which are novel finding, and their abundant existence in prostate cancer patients. We demonstrated that the TMPRSS2/ERG fusion genes have biological activities in prostate epithelial cells that can promote tumor initiation and progression and that these activities depend on the isoforms of the fusion gene expressed and ERG splicing exons expressed. The induction of specific shRNA against Type III fusion in VCaP cells dramatically decreased cell proliferation in vitro and tumor weight in vivo. Overall, our observation that the TMPRSS2/ERG fusion genes can promote proliferation, invasion and motility are consistent with our previous clinical observations that TMPRSS2/ERG fusions are associated with more aggressive disease.

# Pleiotropic Biological Activities of Alternatively Spliced TMPRSS2/ERG Fusion Gene Transcripts

Jianghua Wang,[1,3] Yi Cai,[1,3] Wendong Yu,[1,3] Chengxi Ren,[1,3] David M. Spencer,[2] and Michael Ittmann[1,3]

Departments of [1]Pathology and [2]Immunology, Baylor College of Medicine; [3]Michael E. DeBakey Department of Veterans Affairs Medical Center, Houston, Texas

## Abstract

**TMPRSS2/ERG gene fusions are found in the majority of prostate cancers; however, there is significant heterogeneity in the 5′ region of the alternatively spliced fusion gene transcripts. We have found that there is also significant heterogeneity within the coding exons as well. There is variable inclusion of a 72-bp exon and other novel alternatively spliced isoforms. To assess the biological significance of these alternatively spliced transcripts, we expressed various transcripts in primary prostatic epithelial cells (PrEC) and in an immortalized PrEC line, PNT1a. The fusion gene transcripts promoted proliferation, invasion, and motility with variable activities that depended on the structure of the 5′ region encoding the TMPRSS2/ERG fusion and the presence of the 72-bp exon. Cotransfection of different isoforms further enhanced biological activity, mimicking the situation *in vivo*, in which multiple isoforms are expressed. Finally, knockdown of the fusion gene in VCaP cells resulted in inhibition of proliferation *in vitro* and tumor progression in an *in vivo* orthotopic mice model. Our results indicate that TMPRSS2/ERG fusion isoforms have variable biological activities promoting tumor initiation and progression and are consistent with our previous clinical observations indicating that certain TMPRSS2/ERG fusion isoforms are significantly correlated with more aggressive disease.** [Cancer Res 2008; 68(20):8516–24]

## Introduction

Chromosomal rearrangements resulting in gene fusions and expression of functional proteins are common in nonepithelial malignancies (1). For certain malignancies, such as chronic myelogenous leukemia, the presence of the fusion gene (BCR-ABL) is critical for diagnosis and the fusion gene protein product is a key therapeutic target. The discovery of recurrent fusion of the androgen-regulated *TMPRSS2* gene to the ETS transcription factors, particularly the *ERG* gene, in the majority of prostate cancer lesions, has led to a paradigm shift in the study of prostate cancer (2). The TMPRSS2/ERG fusion gene occurs in 15% to 80% of prostate cancer lesions, depending on the clinical stage (3–14). A smaller percentage of cases contain fusions with genes for other ETS transcription factors (2, 4, 7, 9, 14–16), often with promoter fusion partners other than TMPRSS2 (17).

The TMPRSS2/ERG gene fusion arises by fusion of the promoter and 5′ portions of the *TMPRSS2* gene (21q22.3) with the coding sequence of the *ERG* gene (21q22.2). Fusion of these two genes occurs by both intrachromosomal deletion and translocation (2, 6, 14, 18). The TMPRSS2 promoter, which contains androgen receptor (AR)-responsive promoter elements (18), can mediate the overexpression of ETS family members in prostate cancer in response to androgens (2). The ubiquitous activity of AR in prostate cancer cells would then result in the constitutive expression of ERG fusion transcripts in the neoplastic prostatic epithelium bearing this fusion gene.

There is significant heterogeneity in the structure of the 5′ end of the mRNA transcripts of the fusion gene (3, 5, 15, 19). Some prostate cancers express a single mRNA isoform, whereas others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We have characterized eight fusion types in prostate cancer (3), which have been confirmed by others (4, 12), and other isoforms have been identified as well. In all cases, the fusion mRNA includes the TMPRSS2 exon 1 and often exon 2 (5, 13). The most common transcript contains the TMPRSS2 exon 1 fused to ERG exon 4, such that translation would have to arise from an internal ATG codon and give rise to a slightly truncated protein, which we have designated as the type III isoform. Of particular interest is an isoform in which TMPRSS2 exon 2 is fused with ERG exon 4 (designated type VI). This variant was present in 26% of our cases with fusion gene expression (3). For this isoform, translation can be initiated from the TMPRSS2 translation initiation codon and results in a true fusion protein containing the first five amino acids of the *TMPRSS2* gene fused to a slightly truncated ERG protein. We found that expression of this isoform is associated with aggressive disease.

The TMPRSS2/ERG fusion can be detected in high-grade prostatic intraepithelial neoplasia (4, 20) and in 40% to 60% of surgically treated prostate carcinomas. These findings argue that the fusion gene plays a critical role in prostate carcinogenesis. ETS transcription factors are generally mitogenic (21) and should promote tumor progression. Most, but not all, studies have shown an association between the presence of the TMPRSS2/ERG fusion and aggressive disease (3, 8, 9, 11–14, 22), although in some studies reverse transcription-PCR (RT-PCR), and not fluorescence *in situ* hybridization, was used to assess fusion status, and it is possible that in some cases only a fraction of genes in the tumor may have fusion gene but generate enough transcript to give a RT-PCR product. We have shown that among cases with the TMPRSS2/ERG fusion, those expressing the type VI isoform were more aggressive than those expressing type III alone (3). Furthermore, some cases expressing only the type III isoform had high levels of fusion gene expression that was also associated with aggressive disease (3).

Recent studies have characterized some of the biological activities of the most common isoform (type III) in benign and

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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transformed prostatic epithelial cells (PrEC; refs. 23, 24). To better understand the biological activities of all of the alternatively spliced TMPRSS2/ERG fusion gene isoforms in prostate cancer, we cloned the variant isoforms described above into expression vectors. During this process, we noted a significant heterogeneity of the coding exons of the fusion gene. Systematic investigation of the biological activities of the various isoforms indicates that they have variable biological activities that can promote tumor initiation and progression, consistent with our previous clinical observations that certain TMPRSS2/ERG fusion isoforms are associated with more aggressive disease.

## Materials and Methods

**Prostate tissue samples.** The clinical and pathologic data for radical prostatectomy cancer samples have been described previously (3). RNAs were prepared as described previously (3).

**Cloning and expression of TMPRSS2/ERG isoforms.** The fusion transcript isoforms were amplified and subcloned into pcDNA 3.1/V5-His-Topo vector (Invitrogen) using patient cDNA samples (3). Primers used are listed in Supplementary Table S1. Primary PrECs were purchased from Lonza and maintained in medium from the same supplier. Fusion isoforms were expressed in PrECs using the retrovirus pBMN-IRES-EGFP, which has a 5′ long terminal repeat driving expression of cDNAs, followed by an IRES and then green fluorescent protein (GFP; ref. 25). High infection efficiency (>80%) was shown in PrECs based on GFP fluorescence, so cells were analyzed without selection. Primers used for the retrovirus construct were 5′-CCGCTCGAGCGCCTAAGCAGGAG-3′ (TMPERG Vir F *Xho*I) and 5′-CCCAGAATGCGGCCGCTTAGTAGTAAGTGCCC-3′ (TMPERG Vir R *Not*I). Amplified fragments were digested by *Xho*I and *Not*I before being ligated into pBMN-I-GFP vector. Retroviruses carrying these two isoforms were generated in Phoenix A packaging cells. PNT1a cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS) and stably transfected with fusion isoforms with a V5 tag, either individually or in combination as described previously (26) followed by G418 selection (100 μg/mL). Pooled cells were used for all experiments. The transcriptional level of the fusion isoforms in both types of cell lines was evaluated by real-time PCR normalized to β-actin as described previously (26).

**Proliferation assay.** Cells ($2.5 \times 10^4$) of each cell line were plated in 35-mm dishes in complete medium. Cells were trypsinized and counted using a Coulter counter at different time points in triplicate. The experiment was repeated thrice.

**Matrigel invasion assay.** The Matrigel invasion assays were performed in triplicate as described previously (26). The experiment was repeated thrice.

**Migration assay.** Motility was assessed using a scratch wound method as described previously (26). This experiment was repeated four times.

**Soft agar colony formation assay.** Dishes (35 mm) with 0.5% base agar layer mixed with 1× culture medium plus 10% FBS were prepared before the seeding of cells. PNT1a cells ($2.5 \times 10^4$) expressing the TMPRSS2/ERG fusion genes or vector controls were plated in 0.35% top agar layer of each agar dish. Plates were stained with crystal violet and cell colonies were counted after incubation at 37°C in humidified incubator for 3 wk. This experiment was repeated twice.

**Western blot and immunoprecipitation.** Western blot was performed as described previously (27). Primary antibodies were anti-V5 monoclonal antibody (1:5,000 dilution; Invitrogen), anti-Flag M2 monoclonal antibody (1:2,000; Stratagene), or anti-β-actin (1:5,000; Sigma). For immunoprecipitation, 1 mg of protein lysate from each sample was incubated with anti-V5 antibody (1:500) for 2 h at 4°C. Then, 20 μL of protein A/G agarose beads (Santa Cruz Biotechnology) were applied to each sample and incubated at 4°C overnight. Pellets were collected by centrifugation at 3,000 rpm for 30 s. Supernatant was carefully aspirated and discarded. Pellets were washed three to four times with radioimmunoprecipitation assay buffer. After the final wash, pellets were resuspensed in 25 μL of 2× Laemmli sample buffer (Bio-Rad) analyzed by standard Western blot protocol (24).

**Generation of short hairpin RNA against type III TMPRSS2/ERG fusion mRNA.** We designed single-stranded primer oligos targeting sequence around the fusion junction site by using Invitrogen's Web site BLOCK-iT RNAi Designer.[4] The primers are 5′-CACCGCGGCAGGAAGCCTTATCAGTTCGAAAACTGATAAGGCTTCCTGCCGC-3′ (shIIITop) and 5′-AAAAGCGGCAGGAAGCCTTATCAGTTTTCGAACTGATAAGGCTTCCTGCCGC-3′ (shIIIBot). The negative control short hairpin RNA (shRNA) target sequence has been described previously (28). Double-stranded oligos were cloned into pENTR/U6 vector containing the U6 promoter and Pol III terminator. pENTR/U6 vector was transferred to plenti6/BLOCKit-DEST vector during LR recombination following the manufacturer's protocol (Invitrogen). Lentivirus was generated in 293FT cells by cotransfection with the packaging mix from Invitrogen and the final plenti6/BLOCKit-DEST expression construct. VCaP cells were infected with lentiviruses and stably selected in 2 μg/mL blasticidin medium.

**VCaP-Luc orthotopic mice model.** pEF1-Luc-IRES-Neo luciferase expression plasmid has been described previously (29). The luciferase-encoding cassette was released by *Nhe*I/*Not*I digestion and inserted into a lentiviral vector pCDH-MCS1-EF1-Puro (System Biosciences) using the same digestion sites to create pCDH-Luc-EF1-Puro. By cotransfection of pCDH-Luc-EF1-Puro with necessary packaging plasmids in 293 cells, lentiviruses were generated. VCaP-sh-III and VCaP-sh-con cells were both infected with lentivirus carrying luciferase and stably selected in puromycin. Eight- to 10-wk-old severe combined immunodeficient (SCID) mice were used for orthotopic injection. VCaP-Luc-sh-III or VCaP-Luc-sh-Con cells ($1 \times 10^6$) were used for each mouse. Total volume of 20 μL was orthotopically injected into the mouse prostate. In each group, 20 mice were used. Tumor growth was assessed at 2, 3, and 4 wk after injection using the IVIS imaging system (Xenogen). Mice were anesthetized and imaged 10 min after D-luciferin (Molecular Probes) injection (25 mg/kg i.p.). Mice were sacrificed at 4 wk after imaging and tumors were collected. Primary tumor weight was recorded and a full necropsy was performed to identify metastasis. Differences in mean tumor size and image signal were examined by *t* test.

**Characterization of ERG isoforms in prostate.** Based on the National Center for Biotechnology Information (NCBI) published sequences and exon analysis through the Ensembl Web site,[5] we aligned 17 ERG exons to the genomic DNA sequence located on Chr.21 25732292-25364248. Primers to amplify ERG isoforms were designed and are listed in Supplementary Table S1. PCR was performed based on a standard protocol and subcloning was done into Topo2.1 vector from Invitrogen. Vectors with insert were sequenced.

## Results

**Alternative splicing of the coding exons of the TMPRSS2/ERG fusion gene.** We have previously identified alternative splicing of the 5′ portion of the TMPRSS2/ERG fusion gene in prostate cancer that was correlated with clinical aggressiveness. To examine the biological functions of the type III and VI TMPRSS2/ERG isoforms, we cloned the coding portion of these variants into expression vector constructs. During this process, we noted the variable presence of a 72-bp fragment by sequence analysis. We have denoted the variants with this fragment, which corresponds to exon 11 of the ERG genomic sequence, type III+72 and type VI+72. To assess the extent of the expression of the 72-bp exon in prostate cancer, we designed an additional primer pair (ERG RT F and ERG RT R; Supplementary Table S1), which spans this region. As shown in Fig. 1*A*, all TMPRSS2/ERG fusion-positive tumor RNAs revealed two bands, the higher band corresponding to the isoform

[4] Invitrogen RNAi Designer Web site: http://rnaidesigner.invitrogen.com/rnaiexpress.

[5] Ensembl Web site: http://www.ensembl.org.

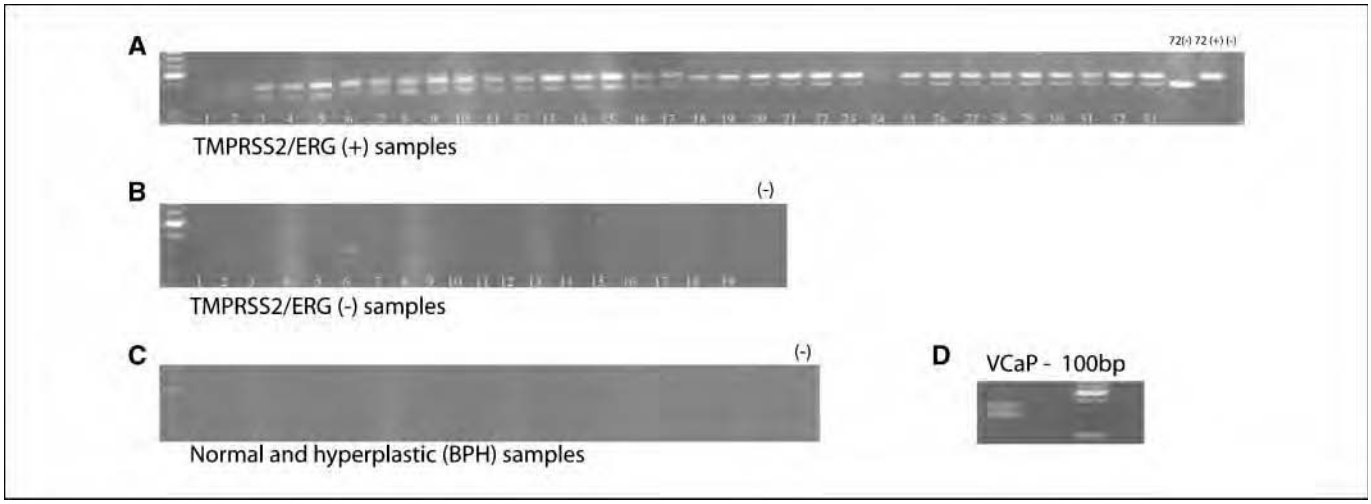


**Figure 1.** Expression of a 72-bp exon in prostate cancer tissues expressing the TMPRSS2/ERG fusion gene. RT-PCR amplification of ERG alternatively spliced isoforms with or without 72-bp exon using primers spanning this exon. *A,* fusion gene–expressing cancer tissues. *B,* fusion-negative cancer tissues. *C,* benign tissues from the peripheral zone (14 tissues) or hyperplastic transition zone (6 tissues), which were free of cancer on pathologic examination. *D,* VCaP cells.

with the 72-bp exon. Fusion-negative cancer RNAs showed rare very weak bands (Fig. 1*B*), whereas benign tissue RNAs were completely negative (Fig. 1*C*). This primer pair detects both the fusion gene and native ERG transcripts and the vast majority of ERG coding transcripts arise from the fusion gene based on comparison of the fusion gene–positive and fusion gene–negative cancers (Fig. 1*A* versus Fig. 1*B*). However, to confirm that expression of the +72-bp exon was present in the fusion transcript, we used another primer pair with forward primer located in the first exon of the TMPRSS2 gene (TMPERG RT-f) and reverse primer within the 72-bp exon (ERG 72R; Supplementary Table S1). All samples were positive (Supplementary Fig. S1*A*). A third primer pair, one located in the TMPRSS2 exon 2 (TMPEx2 F) and the second located in genomic exon 14 (ERG69R2), which will not amplify the type III and III+72 isoforms but will amplify the type VI or VI+72 isoforms, was also used. For the previously identified (3) type VI–positive samples tested, all samples showed double bands with stronger expression of the upper band containing the 72-bp exon (Supplementary Fig. S1*B*). The VCaP cell line expresses both type III and III+72 fusion isoforms (Fig. 1*D*). No other 5′ fusion isoforms, such as the type VI isoform, were identified in VCaP cells.

**Increased cell proliferation in primary and immortalized PrECs expressing TMPRSS2/ERG fusion genes.** A critical step for cancer cell development is the dysregulation of proliferation. Therefore, we initially evaluated the effects of the alternatively spliced fusion gene isoforms on cell proliferation. Using retroviral constructs, we stably expressed TMPRSS2/ERG fusion proteins in primary PrECs. Three groups of PrECs were generated: vector negative control, type III+72, and type (III+72) + (VI+72) groups. The fusion gene transcript levels are shown in Fig. 2*A*. PrECs expressing the type III+72 or type (III+72) + (VI+72) fusion mRNA had significantly increased proliferation rates (Fig. 2*B*) when compared with vector controls ($P < 0.01$, $t$ test; day 9).

To confirm this finding, immortalized normal PrECs (PNT1a) were transfected with plasmids encoding V5 epitope-tagged type III+72, type VI+72, or type (III+72) + (VI+72) fusion genes or the empty vector, and G418-resistant lines were established. As shown in Fig. 3*A*, equivalent expression of the fusion isoforms at the transcriptional level was confirmed by real-time PCR. PNT1a cells expressing the type III+72 isoform alone showed no increase in cell growth, whereas cells expressing the type VI+72 or type (III+72) + (VI+72) isoform had significant increases in growth rate compared with negative control cells (Fig. 3*A*). Interestingly, the PNT1a line

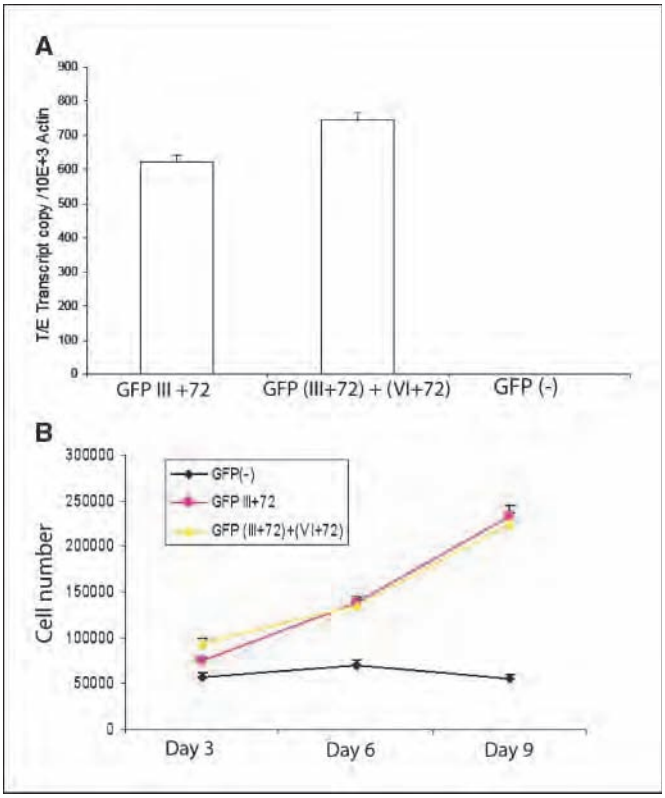


**Figure 2.** TMPRSS2/ERG fusion isoforms increase PrEC proliferation. *A,* expression level of fusion gene in PrECs by real-time PCR, normalized to β-actin. *B,* proliferation of the three groups of PrECs expressing TMPRSS2/ERG fusion type III+72, type (III+72) + (VI+72), or empty vector was measured using a Coulter counter. Cells ($2.5 \times 10^4$) of each cell group were plated in 35-mm dishes in complete medium. Cells were trypsinized and counted at days 3, 6, and 9 in triplicate. *Points,* mean; *bars,* SD.

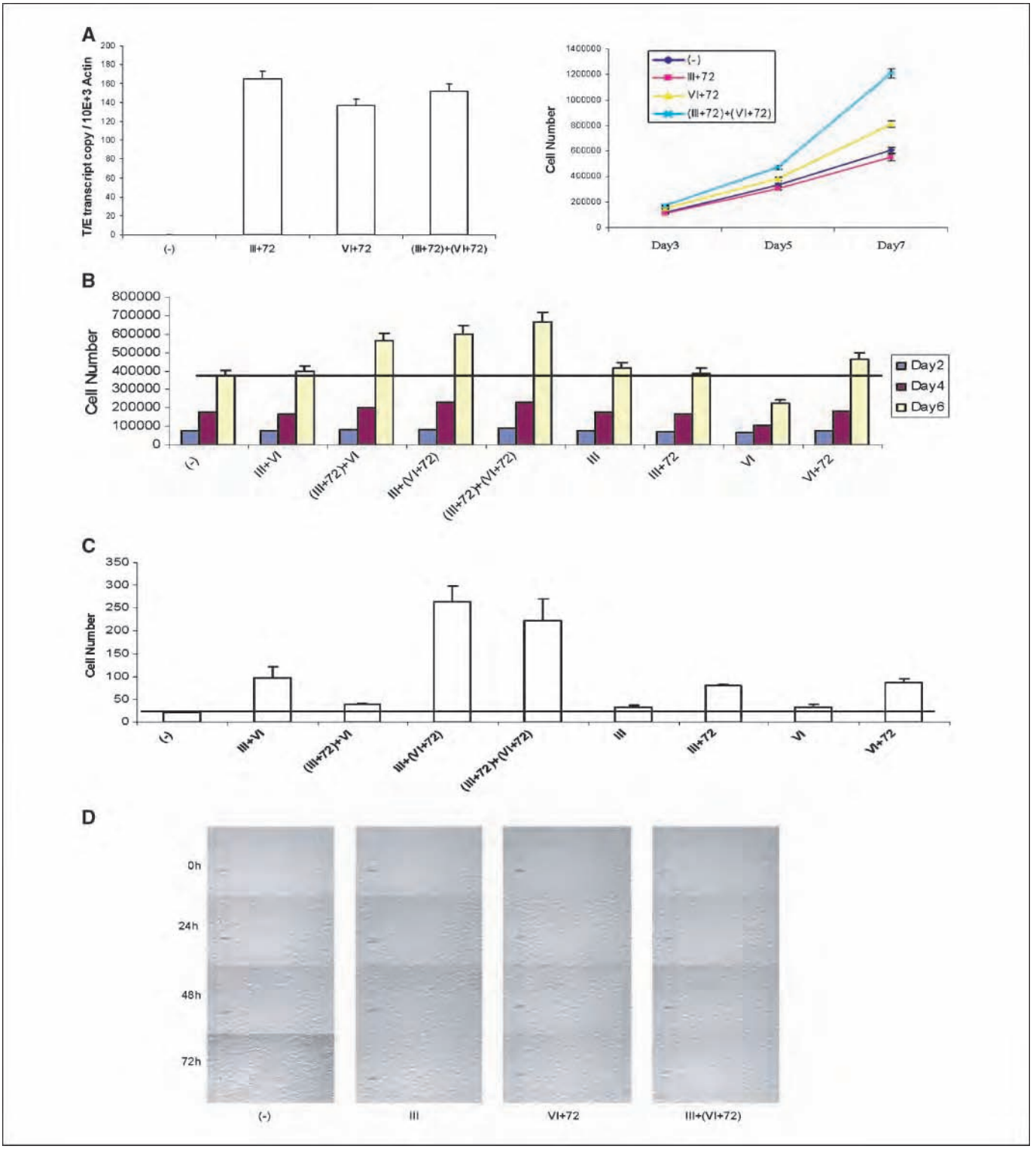


**Figure 3.** TMPRSS2/ERG fusion isoforms affect PNT1a cell proliferation, invasion, and motility. *A,* expression level of fusion gene in PNT1a cells was evaluated by real-time PCR, normalized to β-actin. The growth PNT1a cells expressing TMPRSS2/ERG fusion type III+72, type VI+72, type (III+72) + (VI+72), or empty vector was measured by using a Coulter counter. Cells ($2.5 \times 10^4$) of each cell group were plated in 35-mm dishes in complete medium. Cells were trypsinized and counted at days 3, 5, and 7 in triplicate. *Columns,* mean; *bars,* SD. *B,* proliferation of nine groups of PNT1a cells with overexpression of TMPRSS2/ERG isoforms individually or in combination and control cells. Cell numbers were counted at days 2, 4, and 6. *C,* Matrigel invasion of nine groups of PNT1a cells with overexpression of TMPRSS2/ERG isoforms individually or in combination and control cells. Cells ($2.5 \times 10^4$) of each group were plated into each well at day 0, and after 48 h, the noninvading cells were removed from the upper surface of the membrane and the invading cells were stained and counted under the microscope. Assays were performed in triplicate. *Columns,* mean; *bars,* SD. *D,* PNT1a cells transfected with empty vector or TMPRSS2/ERG fusion types III, VI+72, and III + (VI+72) were seeded at $2.5 \times 10^6$ in 60-mm-diameter culture dishes in complete medium. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS. Complete medium was added and cells were allowed to scatter/migrate into the area of clearing for a total of 72 h and photomicrographs were taken at 0-, 24-, 48-, and 72-h time points. Scratch assays were performed four times and representative results are shown.

expressing both fusion isoforms displayed a 3-fold increase in cell number at day 7 relative to the vector control group ($P < 0.01$, $t$ test) and almost double the cell number versus the group expressing type VI+72 only ($P < 0.01$, $t$ test). To further investigate the potential cooperativity among different isoforms, we overexpressed the four fusion isoforms individually or in combination in PNT1a cells, and proliferation rates were determined in the nine stable PNT1a cell lines (Fig. 3*B*). Equivalent levels of the two fusion isoforms were seen on RT-PCR in all double transfectants, indicating that there is no interference of expression or stability of fusion mRNAs when they are coexpressed (Supplementary Fig. S2). Interestingly, type VI alone seems to even decrease proliferation, perhaps by sequestering factors needed for proliferation. It should be noted that the type VI isoform is never expressed without other isoforms *in vivo* (3). Among four lines expressing individual fusion isoforms, only the cells expressing the type VI+72 isoform showed an increased growth rate relative to the negative control group. Three groups of cotransfected cells, type (III+72) + VI, type III + (VI+72), and type (III+72) + (VI+72), showed significantly higher cell growth rates than the negative control group, which shows synergism between these isoforms in promoting cell proliferation, especially between the type III+72 and VI+72 isoforms.

**Invasion and motility of PNT1a cell lines expressing TMPRSS2/ERG fusion genes.** Another hallmark of neoplastic transformation is the ability to invade the extracellular matrix. We therefore evaluated the invasiveness of the PNT1a cell lines using a Matrigel invasion assay (26). A typical result of one such experiment is shown in Fig. 3*C*. Cells expressing any fusion isoform showed significantly higher invasiveness through Matrigel when compared with vector control PNT1a cells (type III, $P < 0.02$; type VI, $P < 0.04$; all others, $P < 0.01$, $t$ test). Of the cells expressing a single isoform, type VI+72 was most invasive. Of the cells expressing two isoforms, type III + (VI+72) or type (III+72) + (VI+72) fusion isoforms were most invasive. Total cell numbers at 48 h after plating were within 20% of each other (Fig. 3*B*), so that even accounting for these minor differences in cell numbers the invasive ability of all groups was still significantly higher than the vector control cells.

To evaluate cell motility, we performed a wounding assay (26) using type III, type VI+72, type III + (VI+72), and negative control cell lines. In this assay, the ability of cells to migrate and fill a defect in the epithelial monolayer is assessed. As shown in Fig. 3*D*, PNT1a cells expressing the type VI+72 isoform covered the defect within 48 h after the scratch. Cells expressing type III or type III + (VI+72) required 72 h for full closure. The latter result is in contrast to the Matrigel invasion assays, in which the coexpression of the VI+72 isoform with the type III isoform significantly enhanced invasion, and indicates that although motility and invasion *in vitro* have some common pathways, there are almost certainly unique effectors for these two phenotypes as well. All three groups expressing the TMPRSS2/ERG fusion consistently closed the wound quicker than the vector control group, which required >72 h to completely close the defect.

**Colony formation in soft agar of PNT1a cell lines expressing TMPRSS2/ERG fusion genes.** The PNT1a cell line is immortalized but not fully transformed and will not form colonies in soft agar (30). To examine the transforming activity of TMPRSS2/ERG fusion in PNT1a cells, we assessed colony formation in soft agar. No foci were formed in all nine stable cell line groups. As a positive control, we used PNT1a cells expressing Huntingtin-interacting protein 1 (Hip1; ref. 31), which did form colonies in soft agar (data not shown).

**Interactions between TMPRSS2/ERG isoforms.** ETS family transcriptional factors are able to form homodimers or heterodimers. To evaluate whether the alternative fusion gene isoforms showed differences in protein-protein interactions, we transiently cotransfected M2 (Flag)-tagged and V5-tagged fusion gene constructs in 293 cells. Immunoprecipitation was then performed on cell lysates with anti-V5 antibody followed by Western blotting with anti-M2 antibody. As shown in Fig. 4*A*, all isoforms are able to bind to each other and to form homodimers or heterodimers. However, significantly more protein was bound to the type VI+72 than type VI isoform, despite equal protein expression levels (Fig. 4*B–D*), indicating that this 72-bp fragment has potentially important function regulating protein-protein interactions, which may account for its more potent biological activities. No binding to M2-tagged Hip1, which was used as a negative control, was detected.

**Function of the TMPRSS2/ERG fusion in VCaP cells.** VCaP is the only commonly used prostate cancer cell line that expresses the TMPRSS2/ERG fusion gene, specifically the type III and III+72 isoforms. To understand the biological role of the fusion gene *in vivo* in fully transformed cells, we designed a specific oligonucleotide sequence against the type III fusion junction. As shown in Fig. 5*A*, lentiviruses expressing this shRNA can efficiently knock down >60% of type III fusion protein expression when tested in transiently transfected 293 cells by Western blot using anti-V5 antibody. Transcriptional levels of fusion were also assessed in VCaP cells by quantitative real-time PCR using the primers specific for the fusion gene mRNA (3). Similarly, ~60% knockdown efficiency was found in VCaP cells infected with the shRNA lentivirus compared with control VCaP infected with scrambled shRNA and native VCaP cells (Fig. 5*A*). To determine if the fusion gene enhances proliferation in VCaP cells, proliferation was assessed in VCaP expressing shRNA or scrambled control. Decreased cell growth was found in VCaP expressing fusion gene

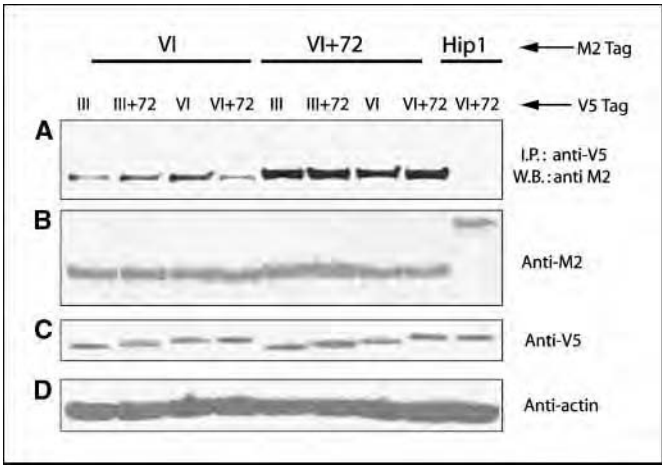


**Figure 4.** Homodimerization and heterodimerization of TMPRSS2/ERG fusion isoforms. *A,* M2 (Flag)-tagged type VI and VI+72 fusion genes or Hip1 protein was cotransfected with four fusion isoforms with a V5 tag in 293 cells and immunoprecipitated with anti-V5 antibody, and Western blot was performed using anti-M2 antibody. Hip1 tagged with M2, cotransfected with type VI+72 with a V5 tag, was the negative control. *B,* expression level of type VI, type VI+72, and Hip1 proteins detected by anti-M2 antibody. *C,* expression level of type III, III+72, VI, and VI+72 proteins detected by anti-V5 antibody. *D,* β-actin input control for each sample.

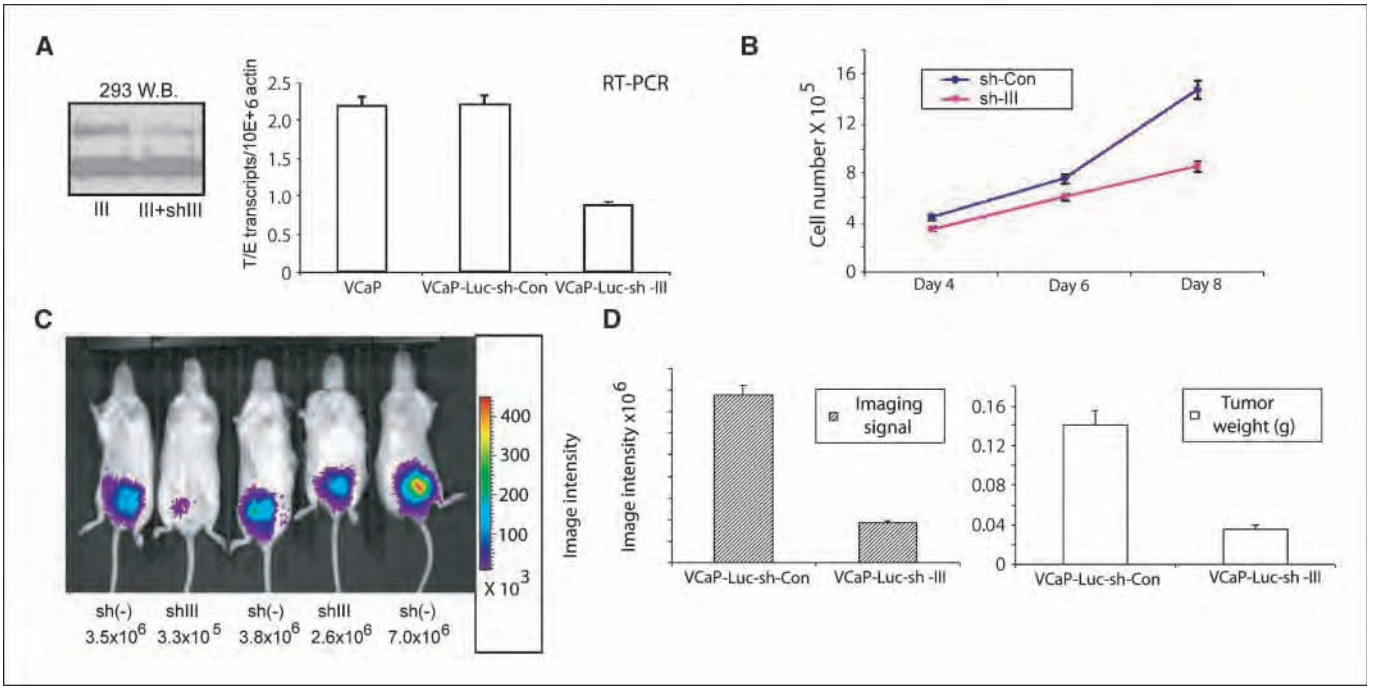


**Figure 5.** Knockdown of the TMPRSS2/ERG fusion in VCaP cells decreases cell proliferation *in vitro* and tumor progression *in vivo*. *A,* 293 cells were transiently transfected with V5-tagged type III fusion gene expression construct and infected with lentivirus carrying shRNA against type III fusion and protein expression was evaluated using Western blotting with anti-V5 antibody. β-Actin loading control is shown. Expression level of fusion gene mRNA in VCaP shRNA negative control or VCaP-sh-III cells by real-time PCR normalized to β-actin. *Columns,* mean; *bars,* SD. *B,* cell growth curve comparing VCaP-Luc-sh-Con and VCaP-Luc-sh-III cell numbers. *Points,* mean; *bars,* SD. *C,* luciferase imaging of tumor growth in live mice from one cage by IVIS imaging system at 4-wk time point. Three control [sh(−)] and two shRNA-expressing (shIII) tumors are shown along with the corresponding image intensity. *D,* luciferase imaging signal at 4 wk after orthotopic injection and tumor weight at same time point.

shRNA compared with the control group ($P < 0.01$; day 8), indicating that these fusion isoforms can affect cell proliferation (Fig. 5*B*). Experiments were repeated thrice with the same result.

To determine if knockdown of the fusion gene expression decreased growth *in vivo*, we used an orthotopic injection model. Orthotopic injection models, in which cells are injected directly into the mouse prostate, have the advantage of developing tumors in the native prostatic microenvironment. We have successfully generated two stable cell lines, VCaP-Luc-sh-III and VCaP-Luc-sh-Con. By using an IVIS imaging system, we were able to monitor the tumor growth in real time (Fig. 5*C*). Luciferase signal was easily detectable at 2 weeks after injection for the majority of mice in the control group. After 4 weeks, the average imaging signal was 4-fold higher in the mice injected with VCaP-Luc-sh-Con cells compared with VCaP-Luc-sh-III cells. Tumor weight was also 4-fold higher in the control group when compared with shRNA-expressing group, consistent with the imaging data (Fig. 5*D*). We examined expression of four genes (*CACNA1D, KCNS3, PLA1A*, and *LAMC2*) that Tomlins and colleagues (23) have shown to be down-regulated by 35% to 70% *in vitro* in their si-ERG–treated VCaP cells by quantitative RT-PCR using mRNAs from seven control and five shRNA-expressing tumors. Three of the four genes identified by Tomlins and colleagues (23) as down-regulated *in vitro* (*CACNA1D, KCNS3, PLA1A*) were also down-regulated *in vivo* by ~50% (data not shown). LAMC2 was only slightly down-regulated (~20%) *in vivo* but was actually down-regulated 60% *in vitro* (data not shown), implying that in some cases alternative pathways for activation of genes may be selected for or activated in tumors in which the fusion gene is down-regulated. The significantly reduced cell growth both *in vitro* and *in vivo* indicates that the TMPRSS2/ERG fusion gene has an important role in regulating primary tumor progression *in vivo*.

**Heterogeneity of coding exons in ERG transcripts in prostate cancer.** To further explore the extent of variability in alternative splicing in the coding exons of the *ERG* gene in prostate cancer, we cloned and sequenced ERG transcripts from human prostate cancer tissues and VCaP cells, almost all of which arise from the fusion gene, using several primer sets designed to detect known ERG isoforms. Nine alternatively spliced ERG isoforms have been reported to date. We carried out an analysis of the NCBI published cDNA sequences and exon analysis through the Ensembl Web site[5] and aligned the ERG exons on the genomic sequence located on Chr.21 from 25732292 to 25364248. Figure 6*A* shows all reported ERG isoforms and their relation to the genomic sequence. Prior reports about TMPRSS2/ERG gene fusion transcripts have all used the ERG2 sequence for reporting the structure of 5′ alternatively spliced TMPRSS2/ERG transcripts and the exon numbers of this isoform are shown. ERG6 and ERG9 were excluded from the study because of their nonfunctional transcripts. Primer pairs were used to amplify different ERG isoforms (Supplementary Table S1), which were cloned and sequenced. Results are summarized in Fig. 6*B*. We found expression of ERG3 with and without the 72-bp exon 11. ERG4 was also expressed. We also detected a novel transcript that contains a previously unreported 61-bp exon. Of note, we were unable to amplify ERG3 or ERG4 transcripts using the exon 17 primer that we used for ERG2. Whether this indicates truncation of these transcripts or a technical problem with the primer pair is currently unclear. We also detected ERG7 with and without the 72-bp exon, as well as a novel isoform missing both genomic exons

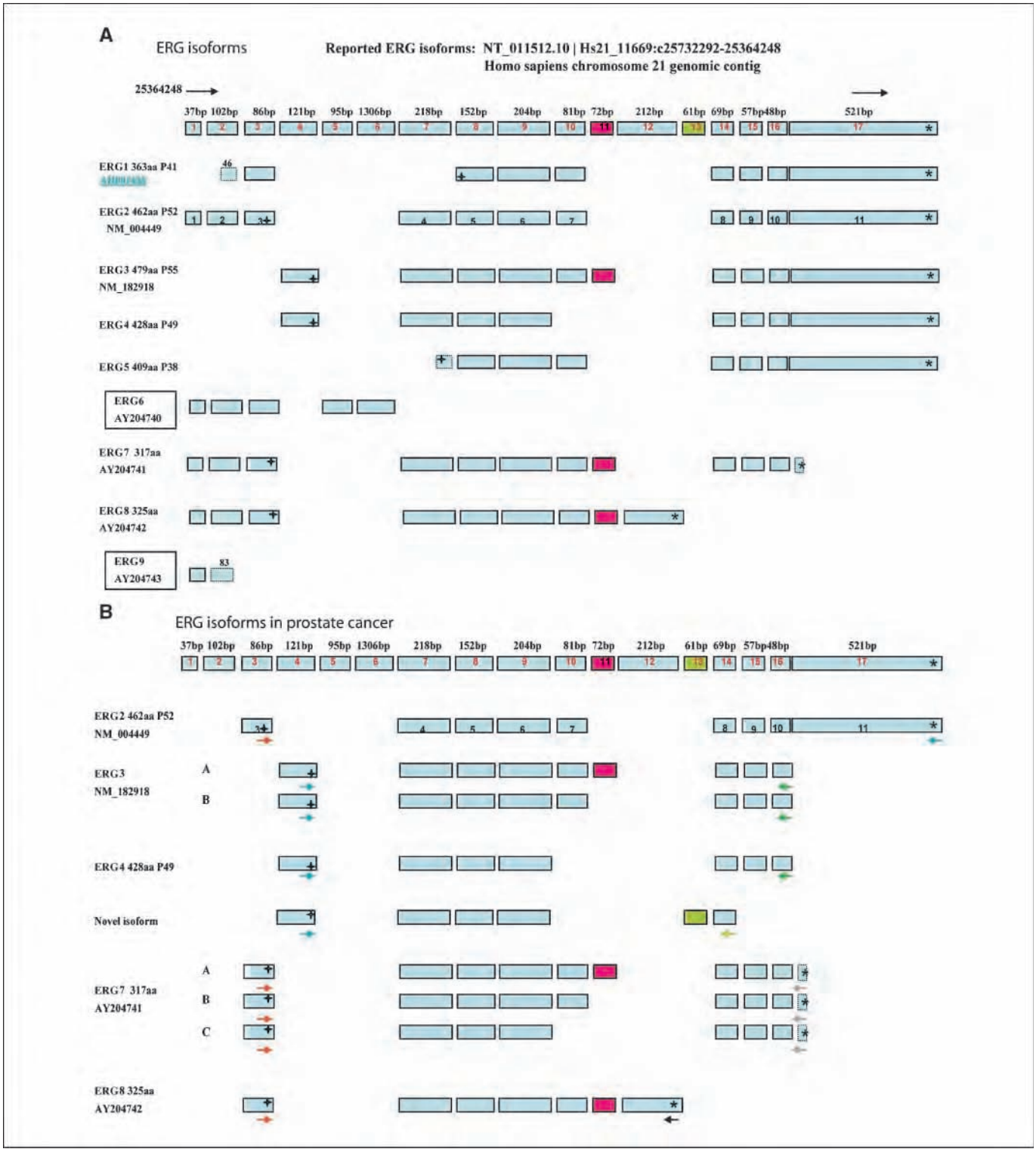


**Figure 6.** Summary of reported ERG transcripts and identified ERG isoforms in prostate. *A,* the nine reported ERG isoforms are listed in the left column with NCBI accession number and predicted protein weight; 17 reported exons are aligned on Chr.21 genomic sequence from 25364248 to 257322921, and the size of each exon is listed on the top of each exon. *Red,* 72-bp exon; *green,* novel 61-bp exon. The 11 exons of ERG2 are indicated. +, in-frame start codons; *, in-frame stop codons. *B,* ERG isoforms in prostate cancer: arrows with different colors stand for different primers used to amplify the ERG transcripts. Subvariants are indicated by letters.

10 and 11. ERG8 was also detected. We did not detect ERG1. Thus, there is significant variability in the coding sequence of the ERG transcripts in prostate cancer as well as in the region of the 5′ fusion.

## Discussion

The discovery that TMPRSS2/ERG gene fusion occurs in 40% to 60% of clinically localized prostate cancers makes this one of the most common genetic lesion in prostate cancer, and elucidating

the clinical and biological consequences of this gene fusion is critical for understanding the pathogenesis of prostate cancer and the development of targeted therapies. We have now shown that TMPRSS2/ERG fusion transcripts have an important biological function in promoting cell proliferation and/or invasion and motility of PrECs and immortalized PrECs. These biological activities are consistent with oncogenic activity of the TMPRSS2/ERG fusion gene in PrECs and indicate that the TMPRSS2/ERG gene fusion is driving neoplastic progression via expression of ERG proteins. This is confirmed by our finding that knockdown of expression of the TMPRSS2/ERG fusion gene decreases primary tumor growth after orthotopic injection of VCaP cells in SCID mice.

Studies by our group and others have shown significant heterogeneity of the alternatively spliced isoforms at the 5′ portion of the TMPRSS2/ERG transcript. We have now shown a similar heterogeneity of the coding sequence. In particular, alternative splicing leading to inclusion or exclusion of a 72-bp exon (genomic exon 11) is common. A recent report (32) using exon arrays to analyze ERG expression in prostate cancer has shown variable expression of this exon in an independent sample set, indicating that alternative splicing at this exon is common enough to lead to detectable alterations in overall expression of this exon. A similar variability in expression was noted for genomic exon 10, and we have noted variable inclusion of this exon in our studies as well. We have also detected other novel isoforms of the TMPRSS2/ERG fusion transcript. The quantitative extent of expression of these alternative isoforms is difficult to assess at present and will require larger-scale sequencing.

Although detection of alternatively spliced transcripts is of interest, a critical question is whether there are differences in the biological activities of these transcripts within benign or transformed PrECs. We have now shown that type VI+72 enhances proliferation of PNT1a cells compared with cells expressing type III+72 at similar transcript levels. This is consistent with our finding that the type VI isoform is associated with more aggressive disease. Similarly, inclusion of the 72-bp exon in type VI significantly enhances proliferation in PNT1a cells relative to type VI without this exon. It should be noted that although the type III isoform without the 72-bp exon has no activity in promoting proliferation, it can enhance both invasion and motility, although less effectively than type VI+72, indicating pleiotropic activities of each specific isoform. Finally, it should be noted that the biological activity of each isoform is related to cellular context. For example, the type III+72 isoform enhances proliferation in PrECs but not in immortalized normal PrECs (PNT1a). PNT1a cells are immortalized with SV40 T antigen and as such have alterations of the p53 and retinoblastoma gene pathways that may affect the biological activities of individual fusion gene isoforms. Thus, alternatively spliced isoforms, both at the 5′ portion of the gene and in the coding exons, have variable, pleiotropic activities that can enhance various aspects of the transformed phenotype depending on the cellular context.

During the analysis of the TMPRSS2/ERG isoforms in prostate cancer tissues, we noted that expression of the type VI isoform was always accompanied by expression of the type III isoform. To determine whether this coexpression had any biological significance or simply was a reflection of the common expression of the type III isoform, we cotransfected PNT1a cells with various combinations of type VI +/−72 bp and type III +/−72 bp and compared them with cells transfected with individual isoforms. At equivalent transcript levels, this resulted in both increased proliferation and invasion. In addition, we showed that these fusion isoforms can form homodimers or heterodimers *in vitro* with different binding strength, which might be partial reason for this variable ability of promoting cell growth and invasion.

The ERG protein is a member of ETS family, and the common feature of ETS family proteins is their DNA-binding ETS domain. The ETS domain can bind to the purine-rich GGA (A/T) core sequence, and these proteins function as transcription factors (21). The ETS domain, the amino pointed domain, and the ERG protein central domain have all been reported to be involved in dimerization (33). The 72-bp exon is located in the central alternative exon region of *ERG* gene, which is directly 5′ of the central domain of the *ERG* gene. The ERG central domain has been shown to act as an inhibitory domain for protein-protein interaction (34). Therefore, the 72-bp exon may be able to change the protein folding structure and affect interaction with other proteins. ERG proteins and other ETS family members can form heterodimeric or homodimeric complexes to regulate their transcriptional activity (21), which is modulated by the competition for homodimerization versus heterodimerization, depending on the relative intracellular concentrations of ETS proteins. Therefore, we posit that heterodimers between these fusion protein isoforms may have more potent transcriptional activity toward critical target genes promoting proliferation and invasion through extracellular matrix compared with either type VI, type VI+72, type III, or type III+72 homodimers. Obviously, this hypothesis requires direct testing and validation.

Recent studies *in vitro* have indicated that expression of ERG and other ETS transcription factors is associated with increased invasion (17, 23, 24) in both PrECs (23) and immortalized PrECs (23, 24) that may, in part, be linked to increased expression of matrix metalloproteinases and activation of the plasminogen activator pathway (23, 24). This is consistent with our finding that all isoforms tested increase invasion. Studies of proliferation have yielded variable results in other studies. Expression of the type III isoform, which probably includes the 72-bp exon, resulted in increased proliferation in BPH1 (24) but not RWPE cells (23), both immortalized normal PrEC lines. Of note, RWPE overexpressing the fusion gene did not form colonies in soft agar, similar to our results in PNT1a cells, or tumors following orthotopic injection (23). Tomlins and colleagues (15) did not see changes in proliferation in PrECs; however, they used transient expression with an adenovirus, whereas we used stable expression with a retrovirus and only observed significant differences after 6 days of growth. Similarly, Tomlins and colleagues (15) did not see decreased proliferation in VCaP after knockdown with small interfering RNA (siRNA) for 3 days; however, by using stable shRNA-expressing cell lines, we were able to examine a longer time course and observed a significant decrease after 8 days. Of note, recent studies by Sun and colleagues (35) using transfection of a siRNA targeting all ERG mRNAs in VCaP cells showed both decreased proliferation *in vitro* and decreased tumorigenicity *in vivo* in a s.c. xenograft model, consistent with our results. Overall, the data indicate that the type III+72 isoform can enhance proliferation in some contexts and that the type VI+72 isoform is even more potent in this regard.

In summary, we have shown complex alternative splicing of the TMPRSS2/ERG fusion gene in prostate cancer, both at the 5′ fusion junction and in the coding exons, which can significantly affect the biological activities of the encoded proteins. Depending on the

isoform and system examined, the TMPRSS2/ERG fusion gene can enhance proliferation, invasion, and motility. Finally, knockdown of the fusion gene in a cancer cell line inhibits primary tumor growth, indicating that the TMPRSS2/ERG fusion gene is a potential therapeutic target, which is present in the majority of prostate cancers.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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